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Salivary Biomarkers Associated with Myocardial Necrosis: Results from an Alcohol Septal Ablation Model

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Abstract

Objective—To determine if salivary biomarkers demonstrate utility for identifying aspects of myocardial necrosis.

Methods—Twenty-one patients undergoing alcohol septal ablation (ASA) for treatment of hypertrophic cardiomyopathy provided serum and unstimulated whole saliva at baseline and incremental time points post-ASA. Samples were analyzed for seven biomarkers related to myocardial damage, inflammation and tissue remodeling using immunosorbent assays. Levels were compared to baseline and levels observed in 97 healthy controls.

Results—Biomarkers of myocardial damage and inflammation (*i.e.*, troponin I, creatine kinase-MB, myoglobin, C-reactive protein) rose in serum 2 to 812-fold after ASA ($p<0.01$). Significant elevations of 2 to 3.5-fold were observed with C-reactive protein and troponin I in saliva ($p<0.02$). Significant correlations between levels in serum and saliva were observed for C-reactive protein, matrix metalloproteinase-9, and myeloperoxidase ($p < 0.001$).

Conclusions—Select salivary biomarkers reflect changes that occur during, and subsequent to, myocardial necrosis caused by ASA.

Keywords

Alcohol Septal Ablation; Biomarkers; Cardiac Biomarkers; Cardiovascular Disease; Coronary; Myocardial Ischemia; Myocardial Necrosis; Saliva

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Saliva is an oral fluid, composed mainly of water, produced and secreted in large part from the acinar and ductal cells of the major and minor salivary glands. Whole saliva derives additional constituents from serum, gingival crevicular fluid and oral mucosal transudate, making it appealing as a potential diagnostic fluid reflective of circulating levels in blood.[1] Several studies report systemic biomarkers appearing in saliva including electrolytes,[2, 3] blood products,[4] enzymes and tissue destruction molecules,[5–9] inflammatory markers,[10–16] as well as proteins putatively associated with deadly diseases.[17–21] However, the clinical utility of salivary diagnostics for the assessment of systemic disease remains elusive.[20, 22–27]

Cardiovascular disease (CVD) is a major cause of mortality in the U.S. and worldwide. More than 17 million people died from CVD in 2008, with the majority due to atherosclerotic events including myocardial infarction.[28, 29] With the extend and importance of this disease, easy, noninvasive and low cost screening methods for the detection of myocardial infarction continue to be sought. One such potential screening fluid is saliva.[30] Support for the use of saliva for the detection of CVD and myocardial infarction is built upon the framework that many serum biomarkers associated with inflammation, atherosclerosis, plaque stability and myocardial damage are known and appear in oral fluids.[4, 9, 31–35] However, only a limited number of studies have demonstrated correlations between serum and salivary biomarkers of CVD.[14, 33, 36] Thus, additional research is required if we are to gain a greater understanding of the clinical utility of saliva compared to serum with respect to CVD and myocardial infarction assessment. This is particularly important for finding new diagnostics for situations where acquisition, processing or analysis of blood is problematic.

In this research investigation, we hypothesized that select salivary biomarkers would correlate with serum biomarkers of CVD in patients with pre-existing heart disease and could yield important diagnostic information regarding the evolution and progression of myocardial necrosis. To test this hypothesis we employed a unique model of myocardial infarction in patients with a pre-existing CVD (*i.e.*, hypertrophic cardiomyopathy [HCM]) who were scheduled to undergo alcohol septal ablation (ASA). This procedure results in a controlled myocardial infarction and subsequent remodeling processes that permit improved hemodynamics and improved patient outcomes.[37, 38] Together, the pre-existing CVD and subsequent kinetic course of myocardial necrosis allowed us to predict that a spectrum of myocardial damage, inflammation and tissue remodeling biomarkers would appear in serum and saliva at baseline, and significant spikes would occur in these biomarker levels during the post-operative phase consistent with myocardial infarction.[39–43] Here this unique study design permitted us to report the novel findings that salivary levels of troponin (Tn)I and C-reactive protein (CRP) reflected changes in serum consistent with myocardial damage, and select salivary biomarkers correlate with serum biomarkers of CVD.

METHODS

Patients

Patients were eligible for study participation if they underwent ASA as treatment for HCM at the University of Kentucky Chandler Medical Center between May 1, 2007 and May 31, 2011. The diagnosis of HCM was based on established clinical and echocardiographic criteria. At our institution, ASA is performed for patients with HCM if they have severe symptoms (dyspnea, New York Heart Association (NYHA) heart failure class III to IV, Canadian Cardiovascular Society (CCS) anginal class 3 to 4, or recurrent exercise-induced syncope and are refractory to medical therapy with left ventricular outflow tract gradients (LVOT) at rest ≥30mmHg or with provocation ≥50mmHg. Exclusion criteria included age less than 18 years, unable or unwilling to provide informed consent or provide samples,

recently treated with chemotherapeutic drugs, anti-organ rejection drugs, or significant immune modulators within the last 3 months or during the course of the study, febrile illness or active infection at the time of enrollment, or were pregnant. Once screened, 21 patients scheduled for echocardiography-guided ASA were enrolled. In addition, a cohort of healthy subjects (*i.e.*, their medical history was negative for CVD and any current or recent cardiac symptoms) was recruited (n=97) to provide baseline comparative samples for study and to validate that sample processing and storage did not produce unexpected abnormalities. Written informed consent was obtained from all patients before their procedure. The study was approved by the University's Institutional Review Board. All study participants were provided financial remuneration for their participation.

Catheter ablation

In all patients, arterial access was obtained from the femoral region and coronary angiography was performed. A coronary guide catheter was positioned in the left main coronary artery ostium and a guidewire was advanced into a proximal septal perforating artery off the left anterior descending coronary artery. Definity® contrast (Lantheus Medical Imaging, North Billerica, MA, USA) was injected during transthoracic echocardiography to assess the localization and extent of the myocardial territory perfused by the target septal branch. A coronary balloon was inflated at the origin of the septal branch and if no leakage occurred and the perfused myocardial segment was considered responsible for the occurrence of the LVOT gradient, approximately 1 mL of 96% ethanol was slowly injected through the balloon into the vessel to be ablated. The balloon was deflated 5 minutes after infusion of alcohol. This procedure was repeated in the same or additional septal perforators until the LVOT gradient decreased to as low a gradient as possible, ideally zero mmHg. After the procedure, patients were monitored for 72 hours to observe for potential adverse events. Follow-up occurred in our cardiology clinic within the next two to four weeks where repeat transthoracic echocardiography was performed.

Specimens

Serum and UWS samples were collected from each subject at baseline and at 8, 16, 24 and 48 hours post-procedure during times predicted to demonstrate myocardial injury, inflammation and tissue remodeling. Unstimulated whole expectorated saliva (5 mL) was collected at each increment according to a modification in the method described by Navazesh.[44] All baseline samples were collected between 8 a.m. and 6 p.m. Subjects rinsed their mouth with tap water, then expectorated whole saliva into sterile tubes containing a protease inhibitor solution (SIGMAFAST, Sigma, St. Louis, MO.) while seated in an upright position. All samples were immediately placed on ice, transported to the laboratory on ice within 10 minutes of collection, centrifuged, separated into aliquots and stored at −80°C until analyzed.

Immunoassays

All samples (n=190 ablation and 194 control) were analyzed in duplicate within three months of storage. Unpublished data from our lab indicate that levels of biomarkers are consistently maintained when stored for three months at −80°C. Levels of the classic cardiac biomarkers cardiac troponin (TnI), creatine kinase (CK)-MB, myoglobin (MYO) and B-type natriuretic peptide (BNP) were analyzed using a Beckman Access in the hospital CLIA certified laboratory. Concentrations of serum CRP, matrix metalloproteinase (MMP)-9, myeloperoxidase (MPO) as markers of inflammation, tissue damage and remodeling were determined in duplicate using Luminex® IS100-based multiplex kits (Millipore, St. Charles, MO, USA) according to the manufacturer's directions in the University of Kentucky Center for Oral Health Research laboratory. Standards were included on all runs and all results are reported within the linearity of the assays.

Statistical analysis

Statistical analyses were performed using the PC SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Continuous demographic variables are presented as mean, standard deviations (SD), and categorical demographic variables as frequencies and percentages. The interval level variables were compared between patients and controls using two-sample t statistics while categorical variables were compared between these two groups using a chi-square statistic or Fisher's exact test. Biomarker levels, both raw scale and log transformed, are presented as mean and SD by visit time. Spearman's correlations were determined for each biomarker in saliva relative to serum at each time point. Biomarker levels were compared between each follow-up time point and baseline using the Wilcoxon signed rank test, and between baseline and the mean of the healthy control level using the Wilcoxon rank sum test. To control the Type I error rate, statistical significance was determined at the $p < 0.01$ level with marginal significance defined by $p < 0.05$.

RESULTS

Of 21 patients screened and enrolled, 19 provided samples and completed the study. Two patients were excluded because their procedure was cancelled. Characteristics of the participants are shown in Table 1. The majority were female, white and non-tobacco users. All were Caucasian and used alcohol infrequently. The majority completed the study without incident, although 8 of 19 patients developed complete heart block in the immediate post-operative period that necessitated placement of a permanent pacemaker. The 97 controls were on average 10 years younger, more racially diverse, and used less tobacco, but more alcohol than the ASA patients.

Myocardial necrosis biomarkers in serum and UWS

Serum markers of myocardial necrosis (TnI, CK-MB, and MYO) rose significantly after alcohol infusion consistent with features of acute myocardial infarction (Fig. 1). Peak serum levels were evident for MYO at 8 hours (11-fold increase compared to baseline), CK-MB at 8–16 hours (70-fold increase), and TnI at 16–24 hours (over 800-fold increase) ($p<0.001$, respectively). Mean BNP levels at baseline were significantly above healthy control values ($p<0.001$), and trended downward after 16 hours.

These same biomarkers showed elevated trends in saliva, although less dramatic (Fig. 2). The mean level of salivary CK-MB drifted upward early ($p>0.05$) and returned to baseline levels thereafter, and remained below healthy control levels at all time points. Salivary MYO levels rose 2-fold above baseline 8 hours after the ablation and was significantly above healthy control levels between 8 and 24 hours ($p<0.01$). Salivary TnI levels were significantly lower than the mean of the healthy controls at baseline ($p<0.004$) and rose significantly above baseline between 24 and 48 hours post-ASA ($p<0.002$). Levels of salivary BNP, in contrast to serum BNP, were significantly lower than the mean of the healthy controls at baseline, then trended upward after 16 hour to levels 2.3-fold above baseline at 48 hours ($p<0.02$).

Biomarkers associated with inflammation, tissue injury and remodeling

Figure 3 shows that serum CRP rose after 8 hours to levels 3.6-fold above baseline at 48 hours ($p<0.001$). Salivary levels of CRP also trended upward during this time frame compared with baseline ($p<0.012$). The remodeling biomarker MMP-9 and neutrophil enzyme MPO rose early in serum, but were less than 2-fold above baseline at their peak ($p>0.05$). In contrast, salivary levels of MMP-9 were elevated above baseline at 16 hours ($p<0.012$), but remained below baseline and the mean control value thereafter. A downward

trend was observed for salivary MPO that was significantly below baseline at 48 hours (p=0.037).

Correlations of biomarkers in serum and saliva

To further investigate which salivary biomarkers demonstrated potential clinical utility, Spearman correlations were computed with respect to the mean biomarker levels in serum and saliva for each time point. Table 2 shows that the strongest correlations between fluids occurred with CRP, MMP-9 and MPO. The correlation coefficients for MPO and MMP-9 were high and ranged between 0.68–0.90 for all time points ($p<0.05$ in each case). Salivary CRP levels correlated well with serum CRP, with correlations being strongest at baseline, 8 and 48 hours (0.66–0.80, $p<0.022$). MYO also produced significant correlation at 16 hours ($r = 0.58$, $p = 0.04$; not shown).

DISCUSSION

We examined the kinetics of serum and salivary biomarkers in patients with HCM following ASA to help determine the clinical utility of salivary biomarkers with respect to CVD and myocardial necrosis. This model provided several advantages for examining our hypothesis. First, the introduction of alcohol during the ablation procedure produces myocardial necrosis and an associated inflammatory response and tissue remodeling events.[45–47] These events produce elevations in the selected serum biomarkers that we hypothesized would appear in saliva. Second, the use of the alcohol septal ablation as a “planned myocardial infarction” provided the advantage of uniformity of timing and the ability to capture bodily fluids at exact time points after the onset of myocardial necrosis.[45–48] Finally, the model afforded the opportunity to compare baseline levels of important CVD biomarkers in HCM with healthy controls. Using this unique study design, we determined that significant differences in biomarker levels in serum and saliva existed between healthy controls and patients with HCM at rest. Also, we identified salivary biomarkers of potential utility for the diagnosis of acute myocardial infarction, and perhaps determined insight into the “ischemic time” amongst patients presenting with spontaneous myocardial infarction.

During the hours following the ablation we observed a classic rise in serum cardiac enzymes consistent with the picture of spontaneous myocardial necrosis as observed by Hage et al.[39] Hage et al. previously demonstrated that this rise in serum markers of myocardial damage following ASA correlates with the size of myocardial infarction.[39] Like Hage et al., we observed that serum levels of TnI rose approximately 800-fold over baseline and CK-MB rose about 70-fold post-ASA. We also observed the novel finding that MYO rose 11-fold and was an early serum biomarker of myocardial damage post-ASA. Serum levels of CK-MB peaked between 8 and 16 hour and TnI peaked at 24 hour. In comparison, the rise of these biomarkers in saliva was less dramatic and required more time to become significantly elevated above baseline. Here salivary levels of TnI and MYO were significantly elevated above baseline and healthy control levels, respectively at 16 hour. Also, more than 70% of the participants showed elevations in one or more of myonecrosis biomarkers (*i.e.*, CK-MB, MYO and TnI) in saliva in the post-operative phase (data not shown). Together these findings suggest that as a salivary panel CK-MB, MYO and TnI may have utility in screening for myocardial necrosis during situations when acquisition, processing or analysis of blood is problematic such as in military field situations, primary care offices or ambulances. In addition, a benefit of these studies could be the development of portable diagnostic devices that would use small quantities of fluid such as either capillary blood or saliva. Here future studies would establish which fluid demonstrates clinical utility for optimal diagnostics.

We also examined levels of CRP, MMP-9 and MPO. These biomarkers are associated with inflammation, tissue damage and tissue remodeling during and/or subsequent to myocardial infarction. Specific to our study design, serum levels of CRP, MMP-9 and MPO have shown significant elevations during the first 48 hours after a myocardial infarction.[49–52] In our study, serum and salivary levels of CRP demonstrated changes in concentrations in serum after the ablation procedure. Serum CRP levels rose significantly and progressively suggestive of the myocardial infarction[49] and ongoing inflammation that occurs post-ablation. Salivary CRP levels also rose significantly post-ablation, however the increase in concentrations observed were less than those that occurred in serum. After the first 8 hours following ASA, downward trends were observed with levels of MMP-9 and MPO in both serum and saliva suggestive of the relationship between concentrations in both fluids and the nadir that occurs around 48 hours for these two biomarkers.[51, 52]

Time point correlations were examined in an effort to determine which salivary biomarkers correlated best with serum levels during the 48 hours following ASA. Here salivary levels of CRP, MMP-9, and MPO showed impressive correlations with serum levels, both before and after the invasive procedure. Consistent with our observation, Ouellet-Morin et al. showed a correlation between CRP levels measured in serum and saliva,[14] however this observation was not noted by Dillon et al.[34] Our correlation between salivary and serum levels of MMP-9 as well as salivary and serum levels of MPO appears to be the first report of these findings. MMP-9 is known to be associated with collagen remodeling,[46] and CRP and MPO have been shown to be important for predicting risk of CV mortality.[47] Thus, levels of these three biomarkers alone, or in combination, in saliva may provide insight into serum levels as well as the evolving kinetics following the initiation of myocardial necrosis.

A limited number of other serum CVD biomarkers have been reported to correlate with salivary concentrations.[35] Mirzaii-Dizgah et al. reported correlations between serum and salivary creatine phosphokinase and serum and salivary CK-MB concentrations in two studies of 30 acute myocardial infarction patients.[33, 36] In our study, salivary levels of CK-MB did not correlate with serum levels, and salivary CK-MB levels rose only about 0.4-fold above baseline at 16 hour post-ablation. Although it is not completely clear why there is a discrepancy between our findings and those of Mirzaii-Dizgah et al., it is possible that the small number of patients enrolled in both studies is a factor, or differing demographics and methodologies influenced the outcomes. Alternatively, it is possible that the release kinetics of myonecrosis biomarkers may differ following an infarction caused by alcohol infusion compared to the thromboembolic occlusion of a spontaneous myocardial infarction, however existing evidence suggests that they demonstrate several similarities.[45, 46]

In conclusion, we observed 1) a significant change in concentrations in salivary TnI and CRP compared with baseline post-ASA, 2) three CVD biomarkers (CRP, MMP-9 and MPO) in saliva correlate with serum levels, 3) six of seven biomarkers moved temporally in the same direction as those observed in serum, and 4) several of the biomarker levels changed within the first 24 hour post-ablation. These data suggest that a diagnostic window exists in which saliva may have utility for screening for myocardial infarction. Future studies are needed that include larger patient populations to help advance these findings and for the development of salivary diagnostic devices for potential screening of CVD as an alternative or complement to blood in situations where acquisition, processing or analysis of blood is problematic. Inclusion of large populations should help define the concentration ranges displayed during health versus disease as well as the composition of the biomarker panel needed to provide accurate interpretation of the results.

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Clinical Relevance

Although serum biomarkers remain the gold standard for the assessment of acute myocardial infarction, these findings demonstrate saliva's potential for providing additional diagnostic information with respect to myocardial infarction.

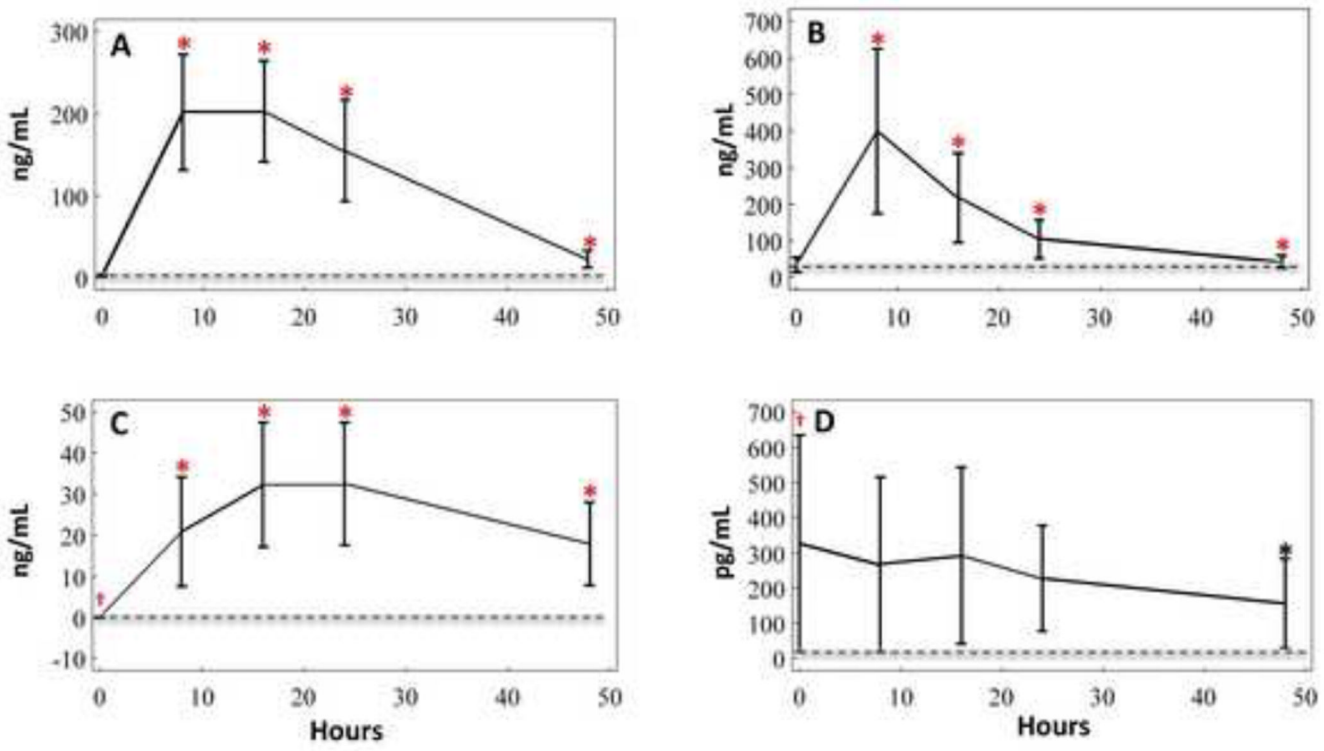

Figure 1.

Serum markers of myocardial damage. Mean concentrations and standard deviations shown of A) CK-MB, B) MYO, C) TnI and D) BNP shown at 0, 8, 16, 24 and 48 hr. Mean healthy control levels shown as dashed lines with standard deviation shown as gray boxes. The red asterisk indicates levels are significantly different from baseline ($p<0.002$) in panels A, B and C, and a black asterisk indicates levels are different from baseline ($p=0.02$) in panel D. The red † indicates levels are significantly different from healthy controls at baseline ($p<0.01$).

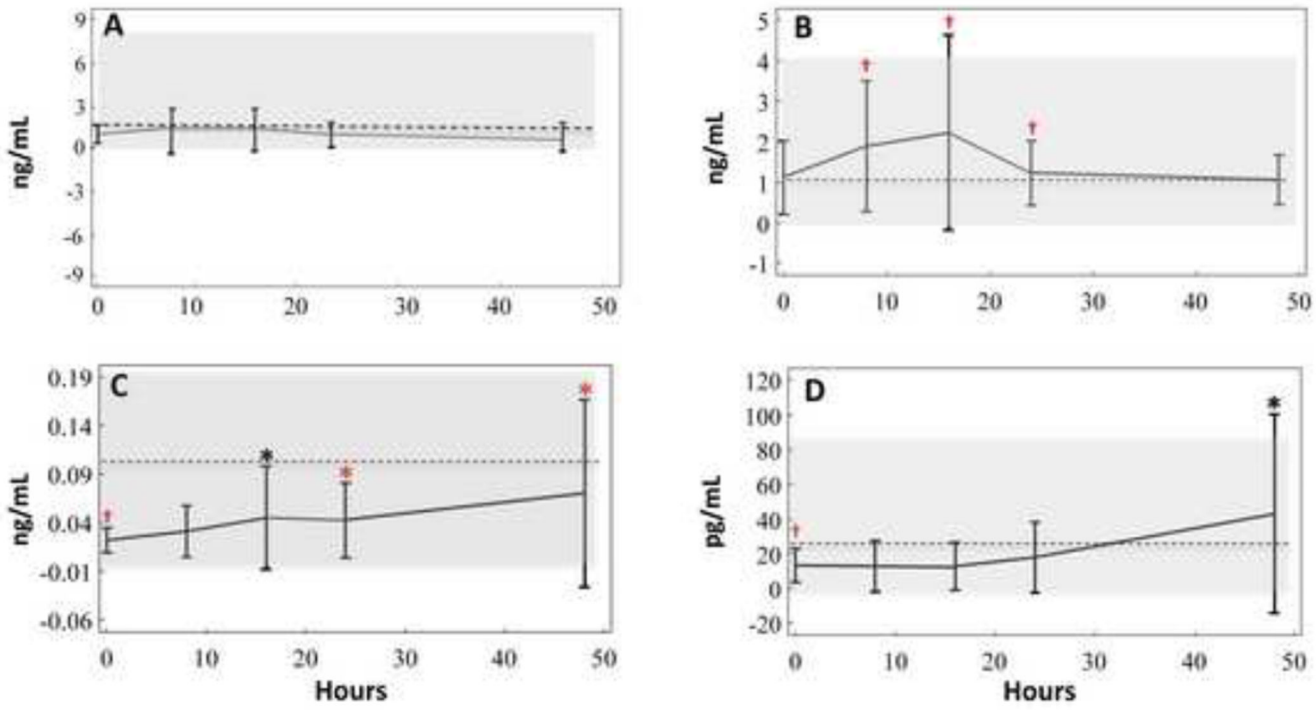

Figure 2.

Salivary biomarkers of myocardial damage. Mean concentrations and standard deviations shown of A) CK-MB, B) MYO, C) TnI, and D) BNP. Mean healthy control levels shown as dashed lines with standard deviation shown as gray boxes. Black asterisk indicates levels are different from baseline ($p<0.02$). The red asterisk indicates levels are significantly different from baseline ($p<0.01$). The red † indicates levels are significantly different from healthy control levels ($p<0.01$). In panel D, the standard deviation of the healthy controls has been truncated; the lower limit is −40

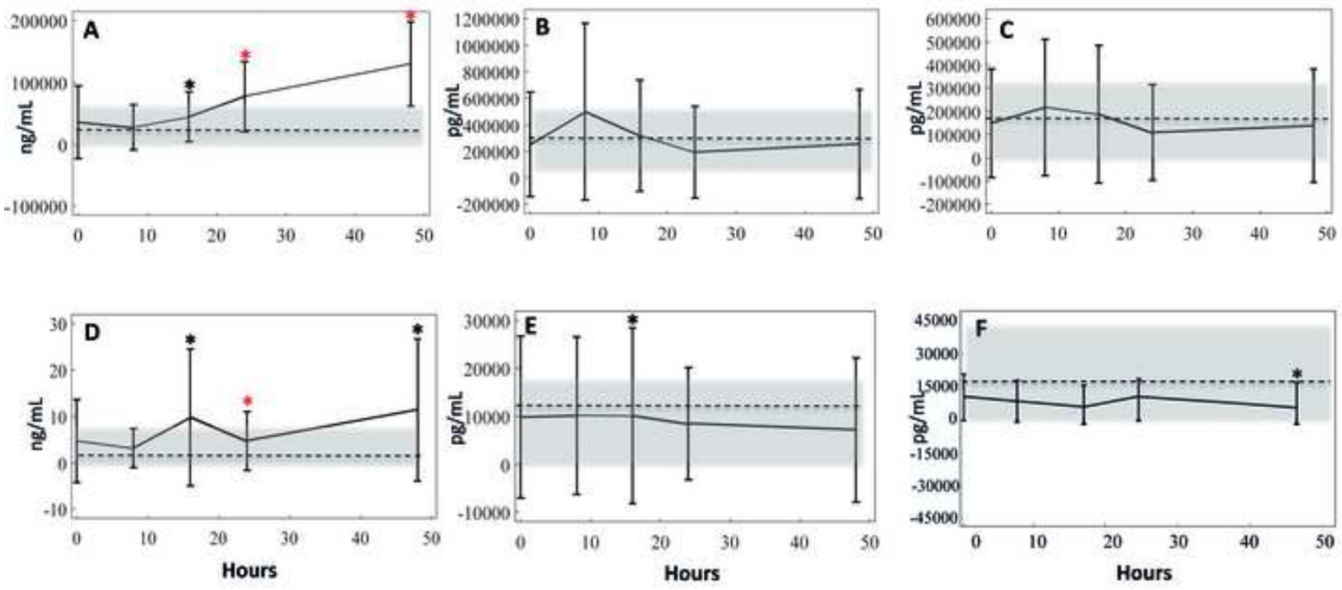

Figure 3.

Biomarkers of inflammation, tissue injury and tissue remodeling. Mean concentrations and standard deviations shown at 0, 8, 16, 24 and 48 hr. A) serum CRP, B) serum MMP-9, C) serum MPO, D) salivary CRP, E) salivary MMP-9 and F) salivary MPO. Mean healthy control levels shown as dashed lines with standard deviation shown as gray boxes. Black asterisk indicates levels are different from baseline ($p \leq 0.04$). The red asterisk indicates levels are significantly different from baseline ($p < 0.01$).

Table 1

Demographics of study population.

	Ablation	Controls
	N=19	N=97
Age (yrs; mean +/− SD)	58.58 +/− 13.41	48.6 +/− 8.6*
Female (%)	63.2	60.8
White (%)	100	86.6
African American (%)	0	8.2
Other races	0	5.2
Current Tobacco Use (%)	47.4	22.7*
Current Alcohol Use (%)	10.5	22.7*
Previous MI (%)	15.8	0*

* $p < 0.05$

Table 2

Biomarkers displaying correlations between serum and saliva levels at multiple time points.

Biomarker	Hour	r	P value
CRP	0	0.80	<0.0001
	8	0.71	0.0217
	16	0.49	0.0899
	24	0.52	0.0491
	48	0.66	0.0108
MMP-9	0	0.78	0.0001
	8	0.68	0.0424
	16	0.76	0.0045
	24	0.74	0.0027
	48	0.71	0.0067
MPO	0	0.72	0.0012
	8	0.85	0.0016
	16	0.90	<0.0001
	24	0.73	0.0030
	48	0.86	0.0001

r = correlation coefficient